MAIL STOP AF
RESPONSE UNDER 37 C.F.R. § 1.116
EXPEDITED PROCEDURE
EXAMINING GROUP 1600

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	H. Lowenheim	Attorney Docket No.:	SOPH116953
Application No.:	09/622,719	Group Art Unit:	1635
Filed:	October 18, 2000	Examiner:	K.A. Lacourciere
Title:	METHOD FOR THE TREATMENT OF DISEASE OR DISORDERS OF THE INNER EAR		

SECOND DECLARATION OF JONATHAN KIL

Seattle, Washington 98101

May 7, 2004

TO THE COMMISSIONER FOR PATENTS:

I, Dr. Jonathan Kil, declare as follows:

1. I am the CEO of Sound Pharmaceuticals, Inc., Seattle, Washington, and I am familiar with the subject matter disclosed and claimed in the above-identified application.

2. A copy of my *curriculum vitae* is appended hereto as Attachment B.

3. I have considered the Office Action dated February 13, 2004, issued in the above-identified application. It is my understanding that the Examiner has rejected claims in the application on the basis of lack of enablement. The Examiner has relied on Pfister & Löwenheim (2002) *Gentherapeutische Aspecte am Innenohr*, pp. 50-7 and Chen & Segil (1999) *Development* 126:1581-90 to conclude that it would take undue experimentation to control the development of sensory cells by administering an inhibitor of cell cycle inhibitors. In addition, the Examiner has relied on Löwenheim et al. (1999) *Proc. Natl. Acad. Sci. U.S.A.* 96:4084-8 to conclude that it is unclear whether release of cells from inhibition of proliferation would initiate the further events required to complete the hair cell regeneration process required to achieve a treatment effect for a disease or disorder of the inner ear.

LAW OFFICES OF
CHRISTENSEN O'CONNOR JOHNSON KINDNESS PLLC
1420 Fifth Avenue
Suite 2800
Seattle, Washington 98101
206.682.8100

4. My colleagues and I conducted the following experiments (1) to assess the proliferation of supporting cells in the organ of Corti and the level of hair cell regeneration in response to aminoglycoside ototoxicity in $p27^{Kip1}$ homozygous mutant mice, and (2) to evaluate the improvement in auditory function in $p27^{Kip1}$ heterozygous mice treated with the ototoxic agent amikacin sulfate.

5. Mouse pups of $p27^{Kip1}$ homozygous null (-/-) received amikacin (1800 mg/kg) from P7 to P12 by subcutaneous (sc) injection once daily. The mitotic tracer 5-bromo-2-deoxyuridine (BrdU) (30 mg/kg, sc) was injected once daily from P10 to P12 simultaneously. Control mice received equivalent injections of BrdU only. Animals were sacrificed 2 days after the last amikacin and BrdU injection.

Mice were decapitated, the cochleae were dissected out and perfused with 4% paraformaldehyde through the round window, then immersed in the same fixative for another 30 minutes. After further dissection, the cochleae were treated with 0.3% hydrogen peroxide in 90% methanol to reduce endogenous peroxidase activity. Cochlea were reacted with 2N HCl to denature the DNA, blocked with 10% horse serum for 1 hour, and incubated with biotin-conjugated sheep anti-BrdU antibody (1:300, BioDesign) overnight at 4°C. After rinsing, the tissues were incubated with an ABC solution (Vector Laboratories), washed, then reacted with diaminobenzidine nickel chloride. Samples were post-fixed with osmium tetroxide (1% in 0.1 M sodium cacodylate buffer), dehydrated through an ethanol series, infiltrated with a mixture of ethanol and historesin, and embedded in historesin. Semi-thin sections (4 microns) were cut and collected. The sections were counter stained with toluidine blue and examined using DIC light microscopy. Each section was examined and each BrdU-positive nucleus was recorded. BrdU-positive nuclei in the same cell type and location in adjacent sections were excluded to avoid double counting.

LAW OFFICES OF
CHRISTENSEN O'CONNOR JOHNSON KINDNESS PLLC
1420 Fifth Avenue
Suite 2800
Seattle, Washington 98101
206.682.8100

6. As shown in Table 1, cell proliferation in the organ of Corti of $p27^{Kip1}$-/- mice was increased after aminoglycoside ototoxicity. 831 sections from an amikacin/BrdU-treated cochlea and 990 sections from a BrdU-treated cochlea were analyzed individually. Cell type determination was based on both morphology and location within the organ of Corti. Normalized values reflect the number of cells expected in the organ of Corti based on 1000 sections at 4 micron thickness.

BrdU-positive nuclei were observed in both hair cells and supporting cells. The number of BrdU-positive inner hair cells (IHCs) and outer hair cells (OHCs) increased following amikacin treatment. An increase in proliferation was also observed in inner phalangeal, Deiter's, and Hensen cells. These data indicate that many of the supporting cell types in the organ of Corti exhibit increases in proliferation in response to amikacin treatment and that the level of hair cell regeneration also increases in response to aminoglycoside ototoxicity.

7. To measure the improvement in auditory function in amikacin sulfate-treated $p27^{Kip1}$ heterozygotes, mice (P7-P12) received systemic injections of amikacin sulfate (500mg/kg/d/s.c) for six consecutive days. Auditory function was measured two weeks and four weeks after amikacin sulfate treatment using the auditory brainstem response (ABR) using subcutaneous recording electrodes placed on three head points in isofluorane-anesthetized mice. The sound intensity threshold was determined by presenting single frequencies as different sound intensities (intensity measured in logarithmic scale or decibels). The higher the tone intensity that is required to elicit the ABR, the higher the auditory threshold, *i.e.*, the worse the auditory function. Significance is determined using one-way analysis of variance (ANOVA) for each stimulus frequency and intensity. Differences are considered statistically significant for p-values <0.05. The data set forth in Table 2 and FIGURE 1 shows auditory improvement in six $p27^{Kip1}$ heterozygote ears compared to four wild-type ears. These results clearly indicate that cells

LAW OFFICES OF
CHRISTENSEN O'CONNOR JOHNSON KINDNESS PLLC
1420 Fifth Avenue
Suite 2800
Seattle, Washington 98101
206.682.8100

released from inhibition of proliferation as a consequence of decreasing $p27^{Kip1}$ gene dosage do initiate and complete the hair cell regeneration process required to achieve an auditory improvement after treatment with an ototoxic agent.

LAW OFFICES OF
CHRISTENSEN O'CONNOR JOHNSON KINDNESS PLLC
1420 Fifth Avenue
Suite 2800
Seattle, Washington 98101
206.682.8100

Table 1. BrdU-Positive Nuclei of Different Cell Types in the Organ of Corti in $p27^{Kip1}$ Mice

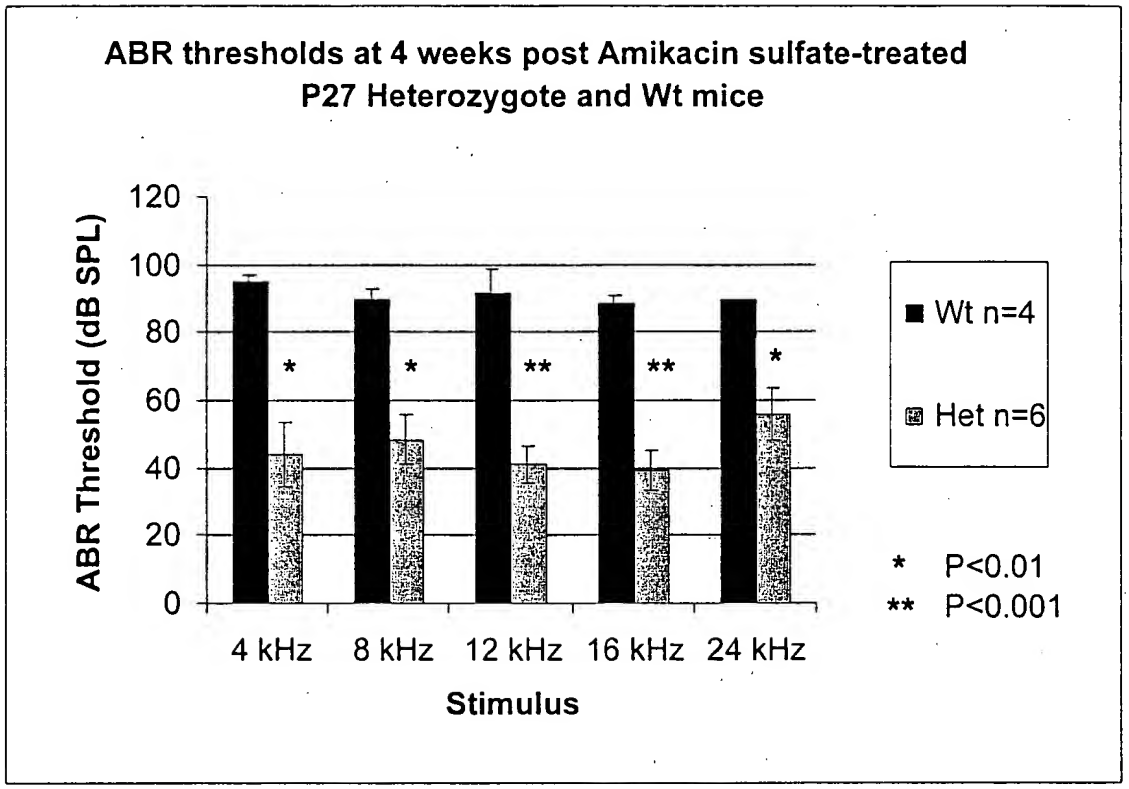
Cell Type	Amikacin +BrdU		BrdU		
	# of BrdU Nuclei	Normalized	# of BrdU Nuclei	Normalized	Exp./Con.
Inner Phalangeal Cells	176	211.8	66	66.7	3.18
Inner Hair Cells	27	32.5	10	10.1	3.22
Pillar Cells	30	36.1	43	43.4	0.83
Outer Hair Cells	5	6	1	1	6
Deiter's Cells	9	10.8	5	5.1	2.12
Hensen Cells	128	154	30	30.3	5.08
Sum	375	451.3	155	156.6	2.88
Sections	831	1000	990	1000	

Table 2. ABR Thresholds at 4 Weeks Post-Amikacin Sulfate Treatment

	4kHz	8kHz	12kHz	16kHz	24kHz
Wildtype Ears					
1	95	85	80	85	90
2	90	95	95	95	90
3	95	85	80	85	90
4	100	95	110	90	90
Mean	95.00	90.00	91.25	88.75	90.00
St. Dev.	4.08	5.77	14.36	4.79	0.00
St. Error	2.04	2.89	7.18	2.39	0.00
$p27^{Kip1}$ Heterozygote Ears					
1	90	80	65	65	90
2	35	45	45	45	35
3	25	40	35	30	45
4	45	60	30	35	50
5	35	35	35	35	60
6	35	30	35	25	55
Mean	44.17	48.33	40.83	39.17	55.83
St. Dev.	23.33	18.62	12.81	14.29	18.82
St. Error	9.52	7.60	5.23	5.83	7.68
P	0.0029	0.0027	0.0004	0.0002	0.0074

LAW OFFICES OF
CHRISTENSEN O'CONNOR JOHNSON KINDNESS PLLC
1420 Fifth Avenue
Suite 2800
Seattle, Washington 98101
206.682.8100

FIGURE 1



LAW OFFICES OF
CHRISTENSEN O'CONNOR JOHNSON KINDNESS PLLC
1420 Fifth Avenue
Suite 2800
Seattle, Washington 98101
206.682.8100

8. All statements made herein and of my own knowledge are true, and all statements made on information and belief are believed to be true; and further, these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful, false statements may jeopardize the validity of the above-identified application or any patent issued thereon.

Respectfully submitted,

Jonathan Kil, M.D.

Date: 5/6/04

KBB:KBB

LAW OFFICES OF
CHRISTENSEN O'CONNOR JOHNSON KINDNESS PLLC
1420 Fifth Avenue
Suite 2800
Seattle, Washington 98101
206.682.8100

JONATHAN KIL, M.D.

Education:

University of Virginia	M.D.	1992-1996
Georgetown University	MD/PhD (candidate)	1990-1992
University of California, Irvine	B.S. in Biological Sciences	1985-1989

Experience:

2001-	Founder, President, CEO Sound Pharmaceuticals Inc., Seattle, WA
1998-2001	Founder, President, CEO, CSO Otogene USA, Inc., Seattle, WA Member of the Vorstand, Otogene AG, Tuebingen, Germany
1996-1998	Senior Fellow Department of Otolaryngology-HNS and VM Bloedel HRC University of Washington
1992-1996	M.D./Ph.D. (candidate) Departments of Neurosciences and Otolaryngology-HNS University of Virginia (UVA)
1990-1992	M.D./Ph.D. candidate (transferred to UVA to conduct inner ear research) Departments of Cell Biology and Otolaryngology-HNS Georgetown University
1989-1990	Research Assistant Department of Anatomy and Neurobiology University of California, Irvine

Grants:

2000-2002	NIH SBIR Phase II #DC04258-02, P.I.
1999-2000	NIH STTR Phase I #DC04258-01, P.I.
1996-1998	Individual NRSA Postdoctoral Research Fellowship #DC00247, P.I.
1991-1992	American Hearing Research Foundation Research Grant, Co-investigator

Awards/Honors:

1995	Association for Research in Otolaryngology Medical Student Travel Award
1995	Winn Medical Student Scholarship for Otolaryngology-HNS
1991	Achievement Reward for College Scientists (ARCS) Foundation Scholarship
1989	Ralph W. Gerard Award for Outstanding Research
1989	Excellence in Research Honors

Publications:

Kil, J., R. Gu, A. H. Sudra, E. D. Lynch, T. Hasson, H. Löwenheim, E. Negrou§, M. L. Fero. (2002) $p27^{Kip1}$ maintains terminal differentiation in the organ of Corti. (submitted)

Kopke, R.D, Coleman, J.K.M., Huang, X., Weisskopf, P.A., Jackson, R.L., Liu, J., Hoffer, M.E., Wood, K., Kil, J., Van De Water, T.R. Novel Strategies to Prevent and Reverse Noise-Induced

Hearing Loss . In Noise Induced Hearing Loss Basic Mechanisms, Prevention and Control. NRN Publishers. 231-253.

Löwenheim, H., D.N. Furness, J. Kil, C. Zinn, K. Gültig, M.L. Fero, D. Frost, A. W. Gummer, J.M. Roberts, E.W Rubel, C.M. Hackney, H.-P. Zenner. (1999) Gene disruption of $p27^{Kip1}$ allows cell proliferation in the postnatal and adult organ of Corti. *Proc. Natl. Acad. Sci. USA*. 96 (7): 4084-8.

H. Löwenheim, J. Kil, P.C. Dartsch, K. Gultig and H. Zenner. 1999. Determination of hair cell degeneration and cell death in neomycin-treated cultures of the rat cochlea. *Hear. Res.* 128: 16-26.

Kil, J., M.E. Warchol and J.T. Corwin. 1997. Cell death, cell proliferation, and estimates of hair cell life spans in the vestibular organs of chicks. *Hear. Res.* 114: 117-126.

Kitzes, L.M., G. H. Kageyama, M.N. Semple and J. Kil. 1995. Development of ectopic projections from the ventral cochlear nucleus to the superior olivary complex induced by neonatal ablation of the contralateral cochlea. *J. Comp. Neurol.* 353:341-363.

Kil, J., G. H. Kageyama, M.N. Semple and L.M. Kitzes. 1995. Development of ventral cochlear nucleus projections to the superior olivary complex in gerbil. *J. Comp. Neurol.* 353:317-340.

Morris, M.S., J. Kil, and M.J. Carvlin. 1993. Magnetic Resonance Imaging of Perilymphatic Fistula. *Laryngoscope*. 103:729-733.

Kil, J. 1989. Developmental plasticity in the gerbil auditory brainstem. J. Undergraduate Research in the Biological Sciences, Univ.of California, Irvine. 19:409-419.

Meetings (selected abstracts):

Kil, J., G.T. Hashisaki, E.W Rubel. (1997) Protection from aminoglycoside ototoxicity by acivicin inhibition of GGT activity. Assoc. Res. Otolaryng. Abs. 20, 100.

Kil, J., Hanigan, M. H., Taylor, Jr., P.T. and Hashisaki, G.T. (1996) Localization of gamma-glutamyl transpeptidase in the chick inner ear sensory epithelia. Soc. Neuro. Abs. 22, 1622.

Memberships:

Association for Research in Otolaryngology
Society for Neuroscience
IBRO
AAAS

Application of new biological approaches to stimulate sensory repair and protection

Matthew C Holley

Institute of Molecular Physiology, Department of Biomedical Sciences, University of Sheffield, Sheffield, UK

The inner ear governs hearing and balance via six sense organs, each composed of a few thousand mechanosensory hair cells. Most inner ear disorders involve irreversible loss of hair cells and their associated nerves. They are a function of age, genetic abnormalities and environmental factors such as noise and the use of ototoxic drugs. The genetics and cell biology of the inner ear have revealed some key molecular mechanisms of development and sensory degeneration that raise hopes for new therapeutic approaches to the regeneration of sensory function. This review highlights these advances and the approaches that might be taken to effect protection and repair. It concludes with the suggestion that we can expect tangible, practical progress towards the clinic over the next 5–10 years and that, to provide the training and skills required to take full advantage of emerging technologies, we should forge much closer links between specialist clinicians and basic scientists.

The challenge of sensory recovery

Hearing loss affects some 9 million people in the UK (<www.defeatingdeafness.org>). One in every 850 babies are born profoundly deaf and progressive or age-related hearing loss affects more than half of all people over 60 years old. In over 80% of cases, the cause is directly or indirectly related to degeneration and death of sensory hair cells and their associated spiral ganglion neurons (SGNs)[1]. The vestibular sensory epithelia are also located within the inner ear and suffer progressive sensory losses that compromise mobility on a similar scale amongst elderly people.

Loss of auditory hair cells in adult mammals is currently irreversible (see Raphael, this volume) and the development of therapeutic techniques designed to replace them is a challenge. Optimism has been fuelled by discoveries that hair cells can be replaced in non-mammalian vertebrates, especially birds, which have provided some of the most promising mechanistic insights[2]. This has been enhanced by evidence for growth of new hair cells in mammalian vestibular epithelia and lies in

Correspondence to: Prof. Matthew C Holley, Institute of Molecular Physiology, Alfred Denny Building, Western Bank, Sheffield S10 2TN, UK

British Medical Bulletin 2002;63: 157–169

the context of the discovery that many tissues, long considered to be irreparable, contain stem cells that can be coaxed towards the task of functional repair[3].

The causes of sensory loss within the inner ear are diverse, but by far the largest number of cases relate to age or to damage caused by noise or ototoxic drugs[4]. The genetic component is highly variable (see Bitner-Glindzicz, this volume). Sadly, numerous drugs used to treat life-threatening illnesses damage hair cells and their innervation directly. These include aminoglycoside antibiotics and drugs such as cisplatin, which is used for cancer treatment. Thus there is a need not only to seek methods of regenerating lost sensory cells but also to develop protective treatments against ototoxic drugs. Noise damage can obviously be avoided using much simpler protective measures such as ear muffs. However, many young people subject themselves to damaging noise levels, which may lead to significant hearing losses later in life.

There are no clinical treatments for the loss of vestibular hair cells. Loss of auditory hair cells, or cochlear function, can be addressed to some extent by insertion of a cochlear implant, which by-passes the sensory cells and delivers electrical stimuli direct to the SGNs within the auditory nerve[5]. Biotechnologies that enhance the interface between the implant electrode and the auditory nerve may widen the candidature for implants and improve the quality of perception[6].

This article addresses the progress in the development of new biotechnologies for treating sensory loss in the inner ear. It does not offer a comprehensive review of the extensive literature, but should highlight research that best represents progress in the selected areas.

Cellular and molecular therapeutic targets

The selection of cellular targets depends upon the nature of the cell loss and whether the aim is to protect or to regenerate tissue. Protection of hair cells or spiral ganglion neurons (SGNs) requires knowledge of the mechanisms of damage and the factors that govern cell survival or death. To replace lost cells, it is necessary to identify potential precursors and to find the means of inducing either appropriate cell division or differentiation. Much of this kind of work is based upon the assumption that an understanding of basic developmental mechanisms will illuminate potential mechanisms for regeneration. Although this applies to a large degree, there are key differences between the two processes, not least because so many developmentally regulated genes are expressed only transiently during development. Added to this, there are significant differences between species and between sensory epithelia *in vitro* and *in vivo*[7]. Whilst identification of molecular targets depends upon a wide

spectrum of experimental preparations, *in vivo* studies with mammals are especially important for progress towards effective clinical application.

Approaches to the protection of hair cells and neurons

Growth factors

The clinical application of growth factors is complicated by wide-spread, potential side-effects and the need to deliver them locally and for long periods[8]. Many neurotrophic factors in man have a serum half-life of minutes or hours, and systemic application requires high dose levels to ensure that effective concentrations reach the desired target. This increases the chances of generating non-specific and undesirable side-effects. However, with respect to protective treatments in the inner ear there are two key advantages. The first is that drugs and other agents, including viral vectors, can be delivered directly into the enclosed environment of the inner ear fluids. The second is that application is required only transiently, during the administration of the ototoxic drug.

A considerable amount of groundwork is required to test applications in animals before proceeding to clinical trials, but current evidence is encouraging. Growth factors can influence cell proliferation, differentiation and survival and numerous studies have been carried out on inner ear epithelia from a variety of different animal species.[9] Much of the work has focused on neurotrophin-3 (NT3) and brain-derived neurotrophic factor (BDNF), which are expressed by hair cells and act on tyrosine kinase receptors (trkB and trkC, respectively) on the sensory neurons[10]. Excitation of these receptors is important for neuronal survival[10,11]. Interestingly, electrical activity in the neurons can induce secretion of BDNF and NT3[12] and it transpires that neuronal survival is dependent upon the presence of hair cells and on electrical stimulation[12–14]. Consequently, the loss of hair cells is associated with a loss of SGNs[15,16].

The progressive loss of SGNs has serious implications for the application of cochlear implants. Although there is little direct evidence from patients for a correlation between implant performance and the degree of innervation[17,18], the timing of implantation appears to be crucial in order to maximize the benefit from the remaining auditory nerve fibres[19]. Thus appropriate drugs that can protect the SGNs may be of potential therapeutic value.

The protective action of growth factors has been assessed in terms of hair cells[20] and nerves[11], but one study showed that a combination of agents directed against both cell types can provide significant functional protection against noise or drug-induced damage[21]. Inner hair cells release glutamate, which excites N-methyl-D-aspartate (NMDA) receptors on the dendrites of the SGNs. NMDA receptors have been implicated in

excitotoxic cell death and may be key mediators of damage induced both by noise or by aminoglycosides. NT3 protects SGNs during aminoglycoside treatment in guinea pigs[22] and an NMDA antagonist helps to preserve hair cell morphology, the Preyer reflex and distortion product oto-acoustic emissions (DPOAEs) following similar treatment in the same animal[23]. Applied together, shortly before noise damage or infusion of amikacin into the perilymph, the NMDA antagonist MK801 and NT3 prevent dendritic swelling, preserve the morphology of hair cells and SGNs and significantly decrease threshold shifts in the auditory brain stem responses (ABR)[21]. This kind of study is particularly important, being conducted *in vivo* with measurements of physiological function.

Shinohara *et al*[6] refined the functional analysis by exploring the protective effects of neurotrophic factors in a guinea pig cochlear prosthesis model. Ears were perfused with 10% neomycin for 48 h to induce hearing loss followed by BDNF and ciliary neurotrophic factor (CNTF) for up to 29 days. A platinum electrode placed about 1.5 mm into the cochlea was used to stimulated the SGNs at weekly intervals before the experiment was terminated to allow the histology of the spiral ganglion to be assessed. There was a significant reduction in the electrically evoked ABR thresholds, which correlated with the density of surviving SGNs. The results are clearly very promising although potential side-effects and the longer term efficacy of the treatment will have to be analysed before clinical trials can be undertaken.

Apoptosis

Hair cells assaulted by aminoglycosides or excessive noise suffer a certain degree of damage and then die by apoptosis[24–27]. Apoptosis is a physiological response that controls cell death and may allow epithelial repair without excessive functional disruption, such as decay of the endocochlear potential. Hair cells can be rescued by blocking apoptotic pathways[28] and the associated intracellular signalling cascade involved also provides suitable molecular drug targets. Hair cells damaged either by the aminoglycoside neomycin or by excessive noise activate the c-Jun N-terminal kinase (JNK) pathway leading to apoptosis[26]. Systemic application of CEP-1347, and agent which inhibits the JNK pathway, to guinea pigs *in vivo*, prior to the ototoxic stimuli, reduces hair cell loss in both auditory and vestibular epithelia and decreases temporary threshold shifts in the ABRs[27]. Studies *in vitro* reveal that CEP-1347 is as effective as NT3 as a survival factor for SGNs[26]. This small molecule can be applied systemically and may reach the target cells more effectively, although concerns about side-effects and long-term efficacy remain. Activation of caspases is an early event in apoptosis and caspase

inhibitors can protect both mammalian[29] and chick[30] hair cells from ototoxic damage.

Other protective agents

Aminoglycoside toxicity involves the formation of free radicals via the formation of iron complexes, a process that can be countered by treatment with antioxidants or iron chelators[31]. Recent clinical trials in China have shown that aspirin can act as an extremely effective therapeutic antioxidant, providing protection from ototoxic doses of aminoglycosides without the need to reduce the serum levels required for disease treatment[32]. The results of this trial are not yet published, but they promise a realistic, inexpensive treatment for one of the world's most common causes of hearing loss.

Approaches to the regeneration of hair cells and neurons

Replacement of lost cells is a more challenging task than protection. In practice, the latter applies where justified clinical intervention leads to predictable damage. In seeking mechanisms and likely drug targets for cell replacement, it is important to identify competent cells and then to find ways of stimulating their division and/or differentiation. Studies in development and regeneration suggest that supporting cells and hair cells share a common precursor and that mature supporting cells can form hair cells either via division or phenotypic conversion. A number of specific transcription factors involved in cell determination and differentiation in the inner ear have been identified. These molecules do not present attractive drug targets. However, components of associated intracellular signalling pathways that can regulate their function may do so, and it is worth exploiting *in vitro* preparations, including cell lines and epithelial or organotypic cultures to this end.

Growth factors and other agents in vivo

There is some potential for stimulating sensory regeneration in vestibular epithelia. Cell proliferation and the appearance of new hair cells in vestibular epithelia from adult rats was induced *in vivo* by infusion of insulin and TGFβ into the cochlear scala vestibuli[7]. The mean numbers of supporting cells and hair cells labelled with tritiated thymidine within a whole utricular macula after 10 days of treatment was small, only 15 and 3, respectively. The response was enhanced by simultaneous application of gentamicin. Interestingly, similar treatment of vestibular epithelia *in vitro*

did not induce proliferation. This may be due to the fact that growth factor responses are integrated with signalling events via the extracellular matrix[9], and it highlights the problems of correlating data from experiments *in vitro* and *in vivo*. It has also been found that insulin and TGFβ induced or enhanced proliferation in most of the surrounding, non-sensory epithelia, including the transition zone, roof of the membranous labyrinth, underlying stroma and squamous mesothelium[33].

The potential side-effects of growth factor therapy may be subtle, but if detrimental effects occur within the inner ear then they may compromise functional recovery. This issue has been addressed by measuring horizontal vestibular-ocular reflexes (HVORs) in adult guinea pigs following treatment with gentamicin[34]. It is significant that in this study the growth factor treatment, consisting of TGFα, IGF-1, retinoic acid and BDNF, was delivered 7 days after the gentamicin and *via* an infusion pump directly into the vestibule. A recovery in HVORs was associated with hair cell recovery, including type 1 cells in the cristae. This is a step forward, but problems relating to the complexity of the growth factor infusion and the longer term stability of recovery still need to be addressed.

The machinery of cell proliferation

Low levels of cell division and hair cell replacement occur in mammalian vestibular epithelia following hair cell loss[35]. Experiments *in vitro* show that small numbers of cells in utricular sensory epithelia from guinea pigs and humans enter S-phase following treatment with aminoglycosides[36]. The effects can be enhanced with various combinations of growth factors[37]. Regeneration of hair cells in the auditory epithelia of birds very obviously involves a proliferative response, which is mediated by cAMP[38]. The intracellular signalling pathways activated by the human recombinant glial growth factor 2 (rhGGF2) have been studied in supporting cell preparations derived from rat utricles[39,40]. Brief exposure to forskolin, which activates adenylyl cyclase and increases cAMP, stimulates some cells to enter the cell cycle. The response is clearly increased in the presence of rhGGF2 and is blocked by inhibitors of membrane receptor recycling[40]. The effects of rhGGF2 involve the activation of phosphoinositol-3-kinase, protein kinase C and mitogen-activated protein kinase and an increase in intracellular calcium, all of which trigger a mitogenic response. It is important to elucidate these intracellular events because the components may offer suitable drug targets. In mammalian epithelia, the cyclin-dependent kinase inhibitor $p27^{kip1}$ is expressed in supporting cells and in null mouse mutants the number of hair cells and supporting cells is greater than normal[41,42]. It may be possible to induce proliferative replacement of hair cells by suppressing p27 or associated proteins in supporting cells.

Conversion of supporting cells to hair cells

There is strong evidence that new hair cells can be derived by phenotypic conversion or transdifferentiation of supporting cells both in birds and mammals. Understanding the molecular mechanisms that underlie this process may reveal potential therapeutic targets. One way of addressing the issue is to screen cultures for molecular events in supporting cells following damage to sensory epithelia[43]. FGF receptors are up-regulated in chick epithelial supporting cells[44,45], and a receptor-like protein tyrosine phosphatase (RPTP) is down-regulated[46]. Down-regulation of RPTP is an early event following ototoxic damage and may be linked to release of an inhibitory influence on cell proliferation[46].

Non-sensory epithelial cells within the greater epithelial ridge (GER) can be converted into *bona fide* hair cells when transfected with the basic helix loop helix (bHLH) transcription factor Math1[47]. Supporting cells and non-sensory epithelial cells are less versatile in adult ears. There is no evidence that Math1 can induce the same response in adult supporting cells or in cells that form the epithelial scar following hair cell loss. This is important because cells induced in this way must be located with some precision within the organ of Corti to detect vibration of the basilar membrane.

Gene therapy

Gene transfer provides a potentially powerful method of addressing a variety of problems. It is conceptually seductive, presenting the opportunity to achieve the ultimate in microsurgery. In principle, cells within the ear can be modified to secrete therapeutic gene products, such as growth factors, and they can be re-programmed to develop different functional properties or to correct an inherent genetic defect[48]. In practice, there are many problems to solve on the roads to clinical application, not least the design and delivery of safe, effective vectors that reliably infect suitable target cells and confer appropriate levels of gene expression over a sufficient period of time.

Adenoviral vectors (AdV) are currently the most efficient and specific means of gene transfer to the inner ear. Non-viral vehicles such as plasmids, liposomes and synthetic polymers are much less efficient and certainly less specific. Amongst the different viral vectors applied either *in vitro* or *in vivo*, herpes simplex virus (HSV), vaccinia, lentivirus and adeno-associated virus (AAV) appear to be less effective[49-51]. HSV may be better for infecting neurons[52], but it is not effective for hair cells[49]. However, the literature should be read with care. Different results may not be due to cell targeting but to delivery and access, especially when

comparing *in vitro* and *in vivo* data, or to viral titres, which vary between different studies. Cell targeting can be controlled by careful selection of the virus and the promoter used to express the transgene[53]. Nevertheless, glial-derived neurotrophic factor (GDNF) delivered *via* AdV vectors can protect both auditory and vestibular hair cells[54,55] as well as SGNs[56] from aminoglycosides.

Viral vectors are potentially toxic and both viral and transgene proteins may generate immune responses *in vivo*. Thus it is important to make functional assessments of hearing and balance when considering clinical application. Such measurements include distortion product oto-acoustic emissions (DPOAEs)[50], hair cell transducer currents[49], Preyer reflexes or balance tasks[57]. Despite the difficulties, current progress suggests that gene therapy mediated by viral vectors is a significant, evolving field with real therapeutic potential. The transfer of genes that encode growth factors is attractive in the sense that, for secreted products, the target cells are less critical. However, cell targeting is essential if genes that regulate cell fate, such as Math1, are to be used directly.

Cell transplantation

Cell transplantation is a rapidly growing research field, particularly in terms of brain repair. It is hard to predict the outcome of transplantation experiments because interactions between cells and tissues *in vivo* are highly complex. Nevertheless, in some cases the behaviour of transplanted cells has been remarkably successful, revealing a plasticity demanding that this area of research be fully explored.

A great deal of progress has been achieved in the visual system where the first clinical trials for cell transplantation are now being considered[58]. In animals, retinal transplantation of conditionally immortal pigment epithelial cells[59] or neural progenitor cells[60] can lead to sensory recovery that is measurable not only in the retina but also in the superior colliculus[59]. The first animal experiments have recently been attempted in the auditory system, involving repair to the central auditory pathway[61]. Embryonic brain tissue was placed into a lesion in the ventral cochlear tract, resulting in some tissue regeneration and associated recovery of the ABR. Controls lacking the embryonic tissue displayed no recovery at all. Neural stem cells derived from the hippocampus have been implanted into the cochlea[62]. It is suggested that some cells adopted hair cell characteristics although the experimental evidence is at a very preliminary stage.

The choice of cells for transplantation is a key issue. Embryonic stem cells (ESCs) could potentially be used if they can be coaxed into a cochlear lineage. For example, they can be induced to differentiate into neuronal phenotypes in the presence of retinoic acid[3]. The task of

inducing auditory sensory or neural phenotypes may be possible with suitable manipulation. An alternative is to isolate cells from the otic placode, otocyst or even at later stages of inner ear development. Several reasonably successful attempts have been made to establish inner ear cell lines[63]. Most lines have been derived from a transgenic animal that carries a conditionally expressed immortalising gene from the SV40 virus. The results show that it is possible to establish conditionally immortal, characterised cell lines that retain an inner ear phenotype. Surprisingly, the cells present realistic candidates for transplantation. Conditionally immortal, embryonic brain cells from the Immortomouse can migrate to sites of damage in the hippocampus and effect both structural and functional repair[64]. This work has led to the development of methods to establish similar conditionally immortalised cells from human tissue. Attempts have been made to identify stem cells from adult, mammalian sensory epithelia[65,66], but this has not yet led to establishment of clonal, characterised cells with predictable phenotypes. The concept of cell transplantation in the ear is thus tangible even if the clinical application remains some way into the future.

Although transplantation of sensory cells into the auditory system seems highly unlikely given the extremely specialised mechanics of the organ of Corti, the task of replacing lost SGNs is more tractable. However, cell replacement is not the only potential application for cell transplantation. Cell lines can be engineered for *ex vivo* gene transfer, that is to deliver secreted products such as growth factors and thus replace neurotrophic functions compromised by the loss of hair cells or supporting cells.

Drug delivery

The method of administering drugs, growth factors or viral vectors to the inner ear depends upon the problem and the desired target cells. It may be systemic[26] or by direct perfusion, often via a mini-pump[67], into cochlear or vestibular chambers[11]. It can also be achieved by diffusion across the round window[68]. The movement of solutes within the inner ear is not uniform and must be modelled carefully to ensure that the appropriate drug doses are applied[69,70]. For example, intratympanic administration of aminoglycosides is used to treat intractable vertigo in patients with Menière's disease[71,72]. At appropriate doses, the drug can target the vestibular epithelia with limited effects on the auditory system. However, functional communication between the cerebrospinal and inner ear fluids can facilitate spread of adenovirus from one innoculated ear to the contralateral ear[73]. These issues are extremely important for any future therapeutic intervention.

Key points for clinical practice

- At present, very few clinicians venture through the oval window. However, the research described in this chapter should hopefully stimulate awareness that many different types of therapeutic intervention are being explored.
- Some interventions may move to clinical trials in the next 5–10 years, others may sink without trace. Regardless of the specific outcome, we should be training more clinicians to understand the structure and function of the inner ear and to take an active part in current research during their training.
- There are numerous potential projects to pursue within a variety of research centres around the world so the trainees of today have every opportunity to equip themselves for future developments.

Acknowledgements

This work was supported by grants from The Wellcome Trust and Defeating Deafness. I thank Andrew Forge, Yehoash Raphael and Marcalo Rivolta for their comments on this manuscript.

References

1 Davis AC. *Hearing in Adults*. London: Whurr, 1995
2 Stone JS, Rubel EW. Cellular studies of auditory hair cell regeneration in birds. *Proc Natl Acad Sci USA* 2000; **97**: 11714–21
3 Guan K, Chang H, Rolletschek A, Wobus AM. Embryonic stem cell-derived neurogenesis. Retinoic acid induction and lineage selection of neuronal cells. *Cell Tissue Res* 2001; **305**: 171–6
4 Forge A, Schacht J. Aminoglycoside antibiotics. *Audiol Neuro-Otol* 2000; **5**: 3–22
5 Rubinstein JT, Miller CA. How do cochlear prostheses work? *Curr Opin Neurobiol* 1999; **9**: 399–404
6 Shinohara T, Bredberg G, Ulfendahl M *et al.* Neurotrophic factor intervention restores auditory function in deafened animals. *Proc Natl Acad Sci USA* 2002; **99**: 1657–60
7 Kuntz AL, Oesterle EC. Transforming growth factor alpha with insulin stimulates cell proliferation *in vivo* in adult rat vestibular sensory epithelium. *J Comp Neurol* 1998; **399**: 413–23
8 Apfel SC. Is the therapeutic application of neurotrophic factors dead? *Ann Neurol* 2002; **51**: 8–11
9 Oesterle EC, Hume CR. Growth factor regulation of the cell cycle in developing and mature inner ear sensory epithelia. *J Neurocytol* 1999; **28**: 877–87
10 Fritzsch B, Pirvola U, Ylikoski J. Making and breaking the innervation of the ear: neurotrophic support during ear development and its clinical implications. *Cell Tissue Res* 1999; **296**: 369–82
11 Miller JM, Chi DH, O'Keeffe LJ, Kruszka P, Raphael Y, Altschuler RA. Neurotrophins can enhance spiral ganglion cell survival after inner hair cell loss. *Int J Dev Neurosci* 1997; **15**: 631–43

12 Hansen, MR, Zha X-M, Bok J, Green SH. Multiple distinct signal pathways, including an autocrine neurotrophic mechanism, contribute to the survival-promoting effect of depolarization on spiral ganglion neurons *in vitro*. *J Neurosci* 2001; **21**: 2256–67

13 Mitchell A, Miller JM, Finger PA, Heller J, Raphael Y, Altschuler RA. Effects of chronic high rate electrical stimulation on the cochlea and eighth nerve in the deafened guinea pig. *Hear Res* 1997; **105**: 30–43

14 Miller AL. Effects of chronic stimulation on auditory nerve survival in ototoxically deafened animals. *Hear Res* 2001; **151**: 1–4

15 Leake PA, Hradek GT, Rebscher SJ, Snyder RL. Chronic intracochlear electrical stimulation induces selective survival of spiral ganglion neurons in neonatally deafened cats. *Hear Res* 1991; **54**: 251–71

16 Dodson HC, Mohuiddin A. Response of spiral ganglion neurones to cochlear hair cell destruction in the guinea pig. *J Neurocytol* 2000; **29**: 525–37

17 Nadol Jr JB. Patterns of neural degeneration in the human cochlea and auditory nerve: implications for cochlear implantation. *Otolaryngol Head Neck Surg* 1997; **117**: 220–8

18 Nadol Jr JB, Shiao JY, Burgess BJ *et al.* Histopathology of cochlear implants in humans. *Ann Otol Rhinol Laryngol* 2001; **110**: 883–91

19 Blamey P, Arndt P, Bergeron F *et al.* Factors affecting auditory performance of postlinguistically deaf adults using cochlear implants. *Audiol Neuro-Otol* 1996; **1**: 293–306

20 Shoji F, Miller AL, Mitchell A, Yamasoba T, Altschuler RA, Miller JM. Differential protective effects of neurotrophins in the attenuation of noise-induced hair cell loss. *Hear Res* 2000; **146**: 134–42

21 Duan M, Agerman K, Ernfors P, Canlon B. Complementary roles of neurotrophin 3 and a N-methyl-D-aspartate antagonist in the protection of noise and aminoglycoside-induced ototoxicity. *Proc Natl Acad Sci USA* 2000; **97**: 7597–602

22 Ernfors P, Duan ML, El-Shamy WM, Canlon B. Protection of auditory neurons from aminoglycoside toxicity by neurotrophin-3. *Nat Med* 1996; **2**: 463–467

23 Basile AS, Huang JM, Xie C, Webster D, Berlin C, Skolnick P. N-methyl-D-aspartate antagonists limit aminoglycoside antibiotic-induced hearing loss. *Nat Med* 1996; **2**: 1338–43

24 Forge A. Outer hair cell loss and supporting cell expansion following chronic gentamicin treatment. *Hear Res* 1985; **19**: 171–82

25 Huang T, Cheng AG, Stupak H *et al.* Oxidative stress-induced apoptosis of cochlear sensory cells: otoprotective strategies. *Int J Dev Neurosci* 2000; **18**: 259–70

26 Pirvola U, Xing-Qun L, Virkkala J *et al.* Rescue of hearing, auditory hair cells, and neurons by CEP-1347/KT7515, an inhibitor of c-Jun N-terminal kinase activation. *J Neurosci* 2000; **20**: 43–50

27 Ylikoski J, Xing-Qun L, Virkkala J, Pirvola U. Blockade of c-Jun N-terminal kinase pathway attenuates gentamicin-induced cochlear and vestibular hair cell death. *Hear Res* 2002; **163**: 71–81

28 Forge A, Li L. Apoptotic death of hair cells in mammalian vestibular sensory epithelia. *Hear Res* 2000; **139**: 97–115

29 Liu W, Staecker H, Stupak H, Malgrange B, Lefebvre P, Van De Water TR. Caspase inhibitors prevent cisplatin-induced apoptosis of auditory sensory cells. *Neuroreport* 1998; **9**: 2609–14

30 Matsui JI, Ogilvie JM, Warchol ME. Inhibition of caspases prevents ototoxic and ongoing hair cell death. *J Neurosci* 2002 **22**: 1218–27

31 Sha SH, Schacht J. Antioxidants attenuate gentamicin-induced free radical formation *in vitro* and ototoxicity *in vivo*: D-methionine is a potential protectant. *Hear Res* 2000; **142**: 34–40

32 Huang W, Chen Y, Zha D *et al.* Prevention of aminoglycoside-induced hearing loss by aspirin: preliminary data from a clinical study. *Assoc Res Otolaryngol Midwinter Meeting* 2002; Abst 262, p 69

33 Kuntz AL, Oesterle EC, Transforming growth factor-alpha with insulin induces proliferation in rat utricular extrasensory epithelia. *Otolaryngol Head Neck Surg* 1998; **118**: 816–24

34 Kopke RD, Jackson RL, Li G *et al.* Growth factor treatment enhances vestibular hair cell renewal and results in improved vestibular function. *Proc Natl Acad Sci USA* 2001; **98**: 5886–91

35 Forge A, Li L, Corwin JT, Nevill G. Ultrastructural evidence for hair cell regeneration in the mammalian inner ear. *Science* 1993; **259**: 1616–9

36 Warchol ME, Lambert PR, Goldstein BJ, Forge A, Corwin JT. Regenerative proliferation in inner ear sensory epithelia from adult guinea pigs and humans. *Science* 1993 **259**: 1619–22

37 Zheng JL, Helbig C, Gao WQ. Induction of cell proliferation by fibroblast and insulin-like growth factors in pure rat inner ear epithelial cell cultures. *J Neurosci* 1997; **17**: 216–26

38 Navaratnam DS, Su HS, Scott SP, Oberholtzer JC. Proliferation in the auditory receptor epithelium mediated by a cyclic AMP-dependent signalling pathway. *Nat Med* 1996; **2**: 1136–9

39 Montcouquiol M, Corwin JT. Brief treatments with forskolin enhance s-phase entry in balance epithelia from the ears of rats. *J Neurosci* 2001; **21**: 974–82

40 Montcouquiol M, Corwin JT. Intracellular signals that control cell proliferation in mammalian balance epithelia: key roles for phosphatidylinositol-3 kinase, mammalian target of rapamycin, and S6 kinases in preference to calcium, protein kinase C, and mitogen-activated protein kinase. *J Neurosci* 2001; **21**: 570–80

41 Lowenheim H, Furness DN, Kil J *et al.* Gene disruption of p27(Kip1) allows cell proliferation in the postnatal and adult organ of Corti. *Proc Natl Acad Sci USA* 1999; **96**: 4084–8

42 Chen P, Segil N. p27(Kip1) links cell proliferation to morphogenesis in the developing organ of Corti. *Development* 1999; **126**: 1581–90

43 Lomax MI, Huang L, Cho Y, Gong TL, Altschuler RA. Differential display and gene arrays to examine auditory plasticity. *Hear Res* 2000; **147**: 293–302

44 Lee KH, Cotanche DA. Localization of the hair-cell-specific protein fimbrin during regeneration in the chicken cochlea. *Audiol Neuro-Otol* 1996; **1**: 41–53

45 Bermingham-McDonogh O, Stone JS, Reh TA, Rubel EW. FGFR3 expression during development and regeneration of the chick inner ear sensory epithelia. *Dev Biol* 2001; **238**: 247–59

46 Kruger RP, Goodyear RJ, Legan PK *et al.* The supporting-cell antigen: a receptor-like protein tyrosine phosphatase expressed in the sensory epithelia of the avian inner ear. *J Neurosci* 1999; **19**: 4815–27

47 Zheng JL, Gao WQ. Overexpression of Math1 induces robust production of extra hair cells in postnatal rat inner ears. *Nat Neurosci* 2000; **3**: 580–6

48 Dazert S, Battaglia A, Ryan AF. Transfection of neonatal rat cochlear cells *in vitro* with an adenovirus vector. *Int J Dev Neurosci* 1997; **15**: 595–600

49 Holt JR, Johns DC, Wang S *et al.* Functional expression of exogenous proteins in mammalian sensory hair cells infected with adenoviral vectors. *J Neurophysiol* 1999; **81**: 1881–8

50 Luebke AE, Steiger JD, Hodges BL, Amalfitano A. A modified adenovirus can transfect cochlear hair cells *in vivo* without compromising cochlear function. *Gene Ther* 2001; **8**: 789–94

51 Staecker H, Gabaizadeh R, Federoff H, Van De Water TR. Brain-derived neurotrophic factor gene therapy prevents spiral ganglion degeneration after hair cell loss. *Otolaryngol Head Neck Surg* 1998; **119**: 7–13

52 Staecker H, Li D, O'Malley Jr BW, Van De Water TR. Gene expression in the mammalian cochlea: a study of multiple vector systems. *Acta Otolaryngol* 2001; **121**: 157–63

53 Luebke AE, Foster PK, Muller CD, Peel AL. Cochlear function and transgene expression in the guinea pig cochlea, using adenovirus- and adeno-associated virus-directed gene transfer. *Hum Gene Ther* 2001; **12**: 773–81

54 Yagi M, Magal E, Sheng Z, Ang KA, Raphael Y. Hair cell protection from aminoglycoside ototoxicity by adenovirus-mediated overexpression of glial cell line-derived neurotrophic factor. *Hum Gene Ther* 1999; **10**: 813–23

55 Suzuki M, Yagi M, Brown JN, Miller AL, Miller JM, Raphael Y. Effect of transgenic GDNF expression on gentamicin-induced cochlear and vestibular toxicity. *Gene Ther* 2000; **7**: 1046–54

56 Yagi M, Kanzaki S, Kawamoto K *et al.* Spiral ganglion neurons are protected from degeneration by GDNF gene therapy. *J Assoc Res Otolaryngol* 2000; **1**: 315–25

57 Dazert S, Aletsee C, Brors D, Gravel C, Sendtner M, Ryan A. *In vivo* adenoviral transduction of the neonatal rat cochlea and middle ear. *Hear Res* 2001; **151**: 30–40

58 Lund RD, Kwan AS, Keegan DJ, Sauve Y, Coffey PJ, Lawrence JM. Cell transplantation as a treatment for retinal disease. *Prog Retin Eye Res* 2001; **20**: 415–49

59 Lund RD, Adamson P, Sauve Y *et al.* Subretinal transplantation of genetically modified human cell lines attenuates loss of visual function in dystrophic rats. *Proc Natl Acad Sci USA* 2001; **98**: 9942–7

60 Young MJ, Ray J, Whiteley SJ, Klassen H, Gage FH. Neuronal differentiation and morphological integration of hippocampal progenitor cells transplanted to the retina of immature and mature dystrophic rats. *Mol Cell Neurosci* 2000; **16**: 197–205
61 Ito J, Murata M, Kawaguchi S. Regeneration and recovery of the hearing function of the central auditory pathway by transplants of embryonic brain tissue in rats. *Exp Neurol* 2001; **169**: 30–5
62 Ito J, Kojima K, Kawaguchi S. Survival of neural stem cells in the cochlea. *Acta Otolaryngol* 2001; **121**: 140–2
63 Rivolta MN, Holley MC. Cell lines from the inner ear. *J Neurobiol* 2002; In press
64 Virley D, Ridley RM, Sinden JD *et al.* Primary CA1 and conditionally immortal MHP36 cell grafts restore conditional discrimination learning and recall in marmosets after excitotoxic lesions of the hippocampal CA1 field. *Brain* 1999; **122**: 2321–35
65 Zhao H-B. Long-term natural culture of cochlear sensory epithelia of guinea pigs. *Neurosci Lett* 2001; **315**: 73–6
66 Malgrange B, Belachew S, Thiry M *et al.* Proliferative generation of mammalian auditory hair cells in culture. *Mech Dev* 2002; 112: 79–88
67 Praetorius M, Limberger A, Muller M *et al.* A novel microperfusion system for the long-term local supply of drugs to the inner ear: implantation and function in the rat model. *Audiol Neuro-Otol* 2001; **6**: 250–8
68 Goycoolea MV. Clinical aspects of round window membrane permeability under normal and pathological conditions. *Acta Otolaryngol* 2001; **121**: 437–47
69 Ghiz AF, Salt AN, DeMott JE, Henson MM, Henson Jr OW, Gewalt SL. Quantitative anatomy of the round window and cochlear aqueduct in guinea pigs. *Hear Res* 2001; **162**: 105–12
70 Salt AN, Ma Y. Quantification of solute entry into cochlear perilymph through the round window membrane. *Hear Res* 2001; **154**: 88–97
71 Hibi T, Suzuki T, Nakashima T. Perilymphatic concentration of gentamicin administered intratympanically in guinea pigs. *Acta Otolaryngol* 2001; **121**: 336–41
72 Hoffer ME, Kopke RD, Weisskopf P, Gottshall K, Allen K, Wester D. Microdose gentamicin administration via the round window microcatheter: results in patients with Menière's disease. *Ann NY Acad Sci* 2001; **942**: 46–51
73 Stover T, Yagi M, Raphael Y. Transduction of the contralateral ear after adenovirus-mediated cochlear gene transfer. *Gene Ther* 2000; **7**: 377–83

```
CLUSTAL W (1.82) multiple sequence alignment


gi|48146914|emb|CR457399.1|          ATGTCAAACGTGCGAGTGTCTAACGGGAGCCCTAGCCTGGAGCGGATGGA 50
gi|516543|gb|U09966.1|MVU09966       ATGTCAAACGTGCGGGTGTCTAACGGGAGCCCGAGCCTGGAGCGGATGGA 50
gi|516545|gb|U09968.1|MMU09968       ATGTCAAACGTGAGAGTGTCTAACGGGAGCCCGAGCCTGGAGCGGATGGA 50
                                     ************ * ***************** ****************

gi|48146914|emb|CR457399.1|          CGCCAGGCAGGCGGAGCACCCCAAGCCCTCGGCCTGCAGGAACCTCTTCG 100
gi|516543|gb|U09966.1|MVU09966       CGCCAGACAGGCGGAGTACCCCAAGCCCTCCGCCTGCAGAAACCTCTTCG 100
gi|516545|gb|U09968.1|MMU09968       CGCCAGACAAGCGGATCACCCCAAGCCTTCCGCCTGCAGAAATCTCTTCG 100
                                     ****** ** *****  ********** ** ******** ** *******

gi|48146914|emb|CR457399.1|          GCCCGGTGGACCACGAAGAGTTAACCCGGGACTTGGAGAAGCACTGCAGA 150
gi|516543|gb|U09966.1|MVU09966       GCCCGGTCAACCACGAAGAGCTGACCCGGGACTTGGAGAAGCACCGCAGA 150
gi|516545|gb|U09968.1|MMU09968       GCCCGGTCAATCATGAAGAACTAACCCGGGACTTGGAGAAGCACTGCCGG 150
                                     *******  * ** *****  * ********************** ** *

gi|48146914|emb|CR457399.1|          GACATGGAAGAGGCGAGCCAGCGCAAGTGGAATTTCGATTTTCAGAATCA 200
gi|516543|gb|U09966.1|MVU09966       GACATGGAAGAGGCAAGCCAGCGCAAGTGGAATTTTGATTTCCAGAATCA 200
gi|516545|gb|U09968.1|MMU09968       GATATGGAAGAAGCGAGTCAGCGCAAGTGGAATTTCGACTTTCAGAATCA 200
                                     ** ******** ** ** ***************** ** ** ********

gi|48146914|emb|CR457399.1|          CAAACCCCTAGAGGGCAAGTACGAGTGGCAAGAGGTGGAGAAGGGCAGCT 250
gi|516543|gb|U09966.1|MVU09966       CAAGCCCCTGGAGGGCAAATACGAGTGGCAGGAGGTGGAGAAGGGCAGCT 250
gi|516545|gb|U09968.1|MMU09968       TAAGCCCCTGGAGGGCAGATACGAATGGCAGGAGGTGGAGAGGGGCAGCT 250
                                      ** ***** *******  ***** ***** ********** ********

gi|48146914|emb|CR457399.1|          TGCCCGAGTTCTACTACAGACCCCCGCGGCCCCCCAAAGGTGCCTGCAAG 300
gi|516543|gb|U09966.1|MVU09966       TGCCGGAGTTCTACTACAGACCCCCGCGGCCACCCAAAGGCGCCTGCAAG 300
gi|516545|gb|U09968.1|MMU09968       TGCCCGAGTTCTACTACAGGCCCCCGCGCCCCCCCAAGAGCGCCTGCAAG 300
                                     **** ************** ******** ** *****  * *********

gi|48146914|emb|CR457399.1|          GTGCCGGCGCAGGAGAGCCAGGATGTCAGCGGGAGCCGCCCGGCGGCGCC 350
gi|516543|gb|U09966.1|MVU09966       GTGCCGGCGCAGGAGAGCCAGGACGTCAGCGGGACCCGGCAGGCCGTGCC 350
gi|516545|gb|U09968.1|MMU09968       GTGCTGGCGCAGGAGAGCCAGGATGTCAGCGGGAGCCGCCAGGCGGTGCC 350
                                     **** ****************** ********** *** * *** * ***

gi|48146914|emb|CR457399.1|          TTTAATTGGGGCTCCGGCTAACTCTGAGGACACGCATTTGGTGGACCCAA 400
gi|516543|gb|U09966.1|MVU09966       TTTAATGGGGTCTCAGGCAAACTCAGAGGACACACACTTGGTAGACCAAA 400
gi|516545|gb|U09968.1|MMU09968       TTTAATTGGGTCTCAGGCAAACTCTGAGGACCGGCATTTGGTGGACCAAA 400
                                     ****** *** *** *** ***** ******   ** ***** **** **

gi|48146914|emb|CR457399.1|          AGACTGATCCGTCGGACAGCCAGACGGGGTTAGCGGAGCAATGCGCAGGA 450
gi|516543|gb|U09966.1|MVU09966       AGACTGACACGGCGGACAACCAGGCTGGCTTAGCGGAGCAGTGCACTGGG 450
gi|516545|gb|U09968.1|MMU09968       TGCCTGACTCGTCAGACAATCAGGCTGGGTTAGCGGAGCAGTGTCCAGGG 450
                                      * ****  ** * ****  *** * ** ********** **  * **

gi|48146914|emb|CR457399.1|          ATAAGGAAGCGACCTGCAACCGACGATTCTTCTACTCAAAACAAAAGAGC 500
gi|516543|gb|U09966.1|MVU09966       ATCAGGAAGCGACCGGCCACAGACGATTCCTCTCCTCAAAACAAAAGAGC 500
gi|516545|gb|U09968.1|MMU09968       ATGAGGAAGCGACCTGCTGCAGAAGATTCTTCTTCGCAAAACAAAAGGGC 500
                                     ** ********** **  * ** ***** *** * ********** **

gi|48146914|emb|CR457399.1|          CAACAGAACAGAAGAAAATGTTTCAGACGGTTCCCCAAATGCCGGTTCTG 550
gi|516543|gb|U09966.1|MVU09966       CAACAGAACAGAAGAAAATGTCTCAGACGGTTCC---------------- 534
gi|516545|gb|U09968.1|MMU09968       CAACAGAACAGAAGAAAATGTTTCAGACGGTTCCCCGAACGCTGGCACTG 550
                                     ******************** ************

gi|48146914|emb|CR457399.1|          TGGAGCAGACGCCCAAGAAGCCTGGCCTCAGAAGACGTCAAACTTAA 597
gi|516543|gb|U09966.1|MVU09966       ----------------------------------------------
gi|516545|gb|U09968.1|MMU09968       TGGAGCAGACGCCCAAGAAGCCCGGCCTTCGA---CGCCAGACGTAA 594
```

```
CLUSTAL W (1.82) Multiple Sequence Alignments


Sequence format is Pearson
Sequence 1: gi|48146914|emb|CR457399.1|         597 bp
Sequence 2: gi|516543|gb|U09966.1|MVU09966      534 bp
Sequence 3: gi|516545|gb|U09968.1|MMU09968      594 bp
Start of Pairwise alignments
Aligning...
Sequences (1:2) Aligned. Score:  89
Sequences (1:3) Aligned. Score:  87
Sequences (2:3) Aligned. Score:  88
Guide tree        file created:   [/ebi/extserv/clustalw-work/interactive/clustalw-2004060
Start of Multiple Alignment
There are 2 groups
Aligning...
Group 1: Sequences:   2      Score:9395
Group 2: Sequences:   3      Score:9773
Alignment Score 10896
CLUSTAL-Alignment file created  [/ebi/extserv/clustalw-work/interactive/clustalw-20040608-
```